Exhibit A

Review

Rosalind E. Jenkins,
Stephen R. Pennington

Department of Human Anatomy
& Cell Biology; University
of Liverpool, Liverpool, UK

Arrays for protein expression profiling: Towards a viable alternative to two-dimensional gel electrophoresis?

Two-dimensional gel electrophoresis (2-DE) is used as a platform method for the measurement of protein expression patterns within cells, tissues or organisms. This approach can support expression profiling of several thousand proteins in multiple samples and as such it is currently unrivalled as a tool for the analysis of protein expression, which is a key component of the rapidly expanding field of proteomics. However, 2-DE has a number of significant limitations and as a consequence, alternative approaches for the measurement of expression of proteins within complex samples are actively being explored. Here we review some existing and emerging methods for protein expression analysis. In particular, we review a range of technologies that might be integrated to support the development of 'arrays' or 'chips' for rapid, high-throughput analysis of protein expression in a manner analogous to the current use of DNA arrays for mRNA expression analysis. We conclude that such separation-independent platforms may ultimately supersede two-dimensional (2-D) gel-based analyses for global protein expression analysis but that before this the technologies might provide important new platforms for diagnostic and prognostic monitoring of diseases.

Keywords: Proteomics / Microarrays / Protein expression / High-throughput diagnostics / Review   PRO 0001

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Correspondence: Dr. Stephen R. Pennington, Department of Human Anatomy & Cell Biology, New Medical School, University of Liverpool, Liverpool L69 3GE, UK
E-Mail: srpennq@liverpool.ac.uk.
Fax: +44-0-151-794-5452

Abbreviations: MIP, molecularly imprinted polymer; **PRM,** protein recognition molecule; **SELDI,** surface-enhanced laser desorption/ionisation; **SPR,** surface plasmon resonance

1 Introduction

Progress in the determination of primary DNA sequence information has had a dramatic impact on the approaches taken to investigate biological organisms. Notably, the completion of several genome sequencing projects, including the genomes of organisms from the three domains of life – archaea [1], prokaryotes [2] and eukaryotes [3–6] – has spawned the fields of comparative and functional genomics in which global approaches are used to examine the function of genes and their encoded proteins [7, 8].

The number and scale of genome sequencing projects continues to increase. There are 40 complete genome sequences in the public domain with a further 127 prokaryotic and 95 eukaryotic genome projects in progress (see Table 1 for websites with up-to-date reports). Perhaps the most exciting sequencing project, that to complete the

Table 1. Web adresses for a selection of sequence databases and analysis tools

Website	Organisation	Information available
ensembl.ebi.ac.uk	European Molecular Biology Laboratory, Heidelberg, Germany Sanger Center, Cambridge, UK	Annotation of human, mouse and worm genomes
genome.wustl.edu/gsc/	Genome Sequencing Centre, Washington University School of Medicine, St. Louis, MO, USA	Human and model organism sequencing projects, EST projects, protocols and technical help
www.hgmp.mrc.ac.uk/	Human Genome Mapping Project Resource Centre, Wellcome Trust Genome Campus, Cambridge, UK	Sequence databases and search engines, phylogenetic linkage analysis, links to useful websites
www.hgsc.bcm.tmc.edu/	Human Genome Sequencing Centre, Baylor College of Medicine, Houston, TX, USA	Human, mouse and *Drosophila* sequencing projects, human transcript database
wit.integratedgenomics.com/GOLD	Integrated Genomics Inc., Chicago, IL, USA	Monitors complete and ongoing genome sequencing projects and links to relevant sites and publications
www.jgi.doe.gov/	Joint Genome Institute, Walnut Creek, CA, USA	Human and microbial sequencing and mapping, functional genomics programme
star.scl.genome.ad.jp/kegg	Kyoto Encyclopaedia of Genes and Genomes, Institute for Chemical Research, Kyoto University, Japan	Sequence databases and current knowledge on molecular interactions
www.ornl.gov/hgmis/	Life Sciences Division, Department of Energy, Oak Ridge National Laboratory, TN, USA	Links to progress reports, publications, meetings *etc*, particularly with regards to the Human Genome Project
www.ncbi.nlm.nih.gov/genome/seq/	National Centre for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA	Sequence, SNP and literature databases, tools for data mining, human and mouse genetic and physical maps
www.sanger.ac.uk/	Sanger Centre, Wellcome Trust Genome Campus, Cambridge, UK	Progress of the human sequencing project plus many of the other prokaryotic and eukaryotic projects
www.tigr.org/tdb/	The Institute for Genomic Research, Rockville, MD, USA	Sequence, function and taxonomy databases for microbes, plants and humans
snp.cshl.org	The SNP Consortium Ltd., Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, USA	SNP map of the human genome
www-genome.wi.mit.edu/	Whitehead Institute Centre for Genome Research, Cambridge, MA, USA	Genetic and physical maps for human, mouse and rat plus human SNP database

human genome, was a much-publicised race between public and privately funded organisations (see Nature commentaries). The publicly funded projects have succeeded in sequencing approximately 65.7% of the 3.1 billion base pairs to date and will produce a complete high quality sequence by 2003 (www.ornl.gov/hgmis). The public projects have already determined the full sequences of chromosomes 5 (ftp.jgi-psf.org/pub/JGI_data/Human/Ch5/Final/), 16 (ftp.jgi-psf.org/pub/JGI_data/Human/Ch16/Final/), 19 (ftp.jgi-psf.org/pub/GJG_data/Human/Ch19/Final/), 21 [9] and 22 [10] via a clone-by-clone approach, providing dramatic evidence of the progress that can be achieved. In June 2000, there was a joint announcement between the public and private efforts of a draft sequence for 90% of the human genome which is now in the process of being assembled (see [198] and www.ornl.gov/hgmis/).

The vast amount of sequence data that is becoming available is being 'mined' by a wide range of computer-based methods in order to predict the function of newly identified genes, and the structure of the encoded proteins [11–19]. In parallel with in silico approaches to examine gene function, methods to undertake comprehensive analysis of gene (mRNA) expression [20–22] and protein-protein interactions have been established [23–25]. Both are currently being applied to genome-wide analysis and are generating massive databases of information. It is very apparent that such 'DNA-based' methods are extremely powerful. Indeed the integration of these methods to analyse gene and protein function is changing fundamentally the way biology is investigated. However, they do not provide an in-depth or global picture of the spatial and temporal patterns of protein expression nor do they reveal the extent to which proteins are post-translationally modified: these are the realm of 'proteomics' [26–30]. Many have attempted to define proteomics [27, 30–32] – for the present purposes, it can be regarded as the analysis of protein expression and activity undertaken in a manner that seeks to maximise the coverage of the analysis.

1.1 Why measure protein expression?

It has become increasingly apparent that the exciting developments in comparative and functional genomics could and should be matched by high-throughput analysis of the protein products of the genes. Clearly, this will be easier to achieve for simpler organisms and realistically may never be achieved within a single platform for more complex organisms such as humans. Initially, the problems with managing the complex and diverse physico-chemical properties of proteins, and the limitations of the methods available, means that proteomics has been

regarded with scepticism by many. More recently this emerging field has increasingly been embraced with enthusiasm and optimism that has in part been justified by some significant 'demonstration' projects [26, 33–35]. The contribution of a proteomics approach is readily apparent when one considers that mRNA expression analyses, though now readily performed with DNA arrays and microarrays, do not always reflect the expression level or activity of the corresponding proteins [36, 37]. Perhaps more importantly, there are biological samples that are not suitable for mRNA expression analysis. For example, biological fluids such as blood plasma, urine, saliva and other excretions/secretions contain proteins and other molecules of physiological and diagnostic significance that require direct measurement and characterisation of their protein content. Thus, further understanding of the biology of organisms, including man, will be best served through the integration of data from DNA, mRNA, protein and metabolic analyses [38–40].

1.2 2-DE as a tool for protein expression profiling

At present, many proteomics projects begin with the fractionation of the cells or tissue under investigation, and subsequent separation of the complex protein components of the samples by 2-DE [32, 41–45]. The 2-DE gels may then be stained to reveal the resolved protein spots, and the gels imaged and compared to identify changes in protein expression between samples [46, 47]. Significant progress has been made in the characterisation and identification of proteins within individual spots, which is now most commonly undertaken by mass spectrometry (MS)-based approaches [48–59]. Potentially, up to 10 000 protein spots may be resolved on a single 2-DE gel [60] so that the method is capable of simultaneously analysing several thousand proteins and at present, this is significantly greater than any orther technique in use. There are now many published reports in which 2-DE has been used very effectively to map large numbers of proteins within samples [35, 47, 61–71] and to identify changes in protein expression [35, 62, 66–68, 70, 72, 73].

Despite the apparent suitability of 2-DE for separation of complex mixtures of proteins, the technique suffers from a number of technical limitations that potentially restrict its long-term use as a platform technology for proteomics. These limitations are well recognised. Briefly, the process is time-consuming, labour-intensive and requires significant technical expertise if quantitatively and spatially reproducible gels are to be generated [45, 74, 75]. At present, the methods for staining proteins within the gels, including fluorescent dyes, reveal only the moderate to high-abundance proteins within a sample and there is a

significant problem in being able to detect proteins over their natural dynamic range of abundance [32, 43, 76]. The molecular weight and p*I* range of proteins that can be examined on a single gel is limited, so that more comprehensive analyses may require the use of multiple gels of an individual sample. Additionally membrane proteins, the targets of up to 80% of therapeutic compounds, are difficult to solubilise for running on 2-DE gels [42], hence the development of methods to support the characterisation of membrane proteins has been identified as an important area for continued improvement in proteomic technologies. The gel images are very complex and their analysis, despite being undertaken by 2-D image analysis software, requires significant and time-consuming manual intervention.

The MS-based identification of protein spots, which can be excised robotically from the gels, has advanced to such a stage that it is amenable to automation [77, 78]. Unfortunately, the full automation of the preceding steps in the process, most notably that of running the 2-DE gels, has not yet been achieved. As might be expected, many are actively seeking solutions to these technical limitations and in some cases significant improvements have been made. However, the limitations are sufficient to have prompted many groups to investigate alternative methods for analysing the proteins present in complex mixtures. The 2-DE based approach to proteomics also

possesses an additional and significant limitation that becomes apparent when it is compared with transcriptomics (Fig. 1). The availability of large numbers of cDNA clones and genome sequences supports the measurement of the expression of large numbers (tens of thousands) of transcripts using DNA arrays [22, 79–86]. Cluster analysis of genome-wide transcript profiling often reveals changes in expression of subsets of the total number of genes analysed [87–91]. Such approaches are beginning to yield important functional data [92]. Furthermore, once changes in mRNA expression between samples have been identified by the use of DNA arrays, it is relatively easy to move to analysis of the smaller number of transcripts on larger numbers of samples (Fig. 1 and 2a). This approach can be used to validate the changes originally observed and establish the extent to which the changes in the expression of individual mRNAs are specific for particular disease states. Potential mechanisms underlying the disease and targets for therapeutic intervention are hence likely to be revealed.

At present, DNA array analyses are undertaken on comparatively small numbers of samples, but the major elements of the process are amenable to automation so that it is relatively easy to envisage how such approaches could be used in the future to analyse large numbers of samples (see Fig. 2b) [82, 93–96]. Furthermore, as noted above, once 'target' transcripts have been identified it is
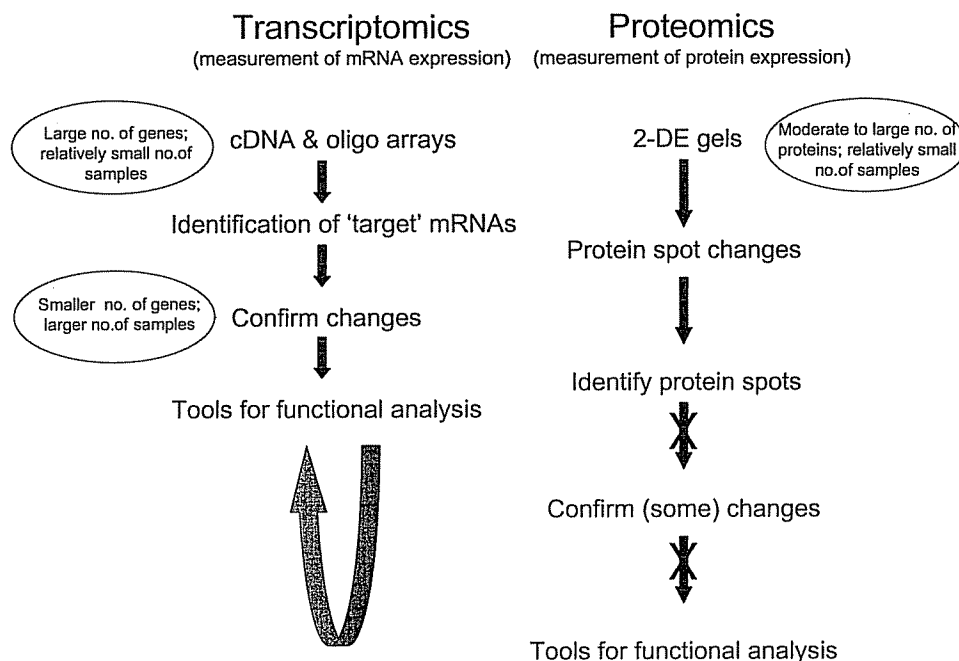

Figure 1. Comparison of transcriptomic *versus* proteomic approaches to gene expression analysis, highlighting the lack of readily available tools to validate protein spot changes on 2-DE gels or to proceed further with the characterisation of selected proteins in the absence of an unequivocal identification from sequence databases.

relatively straightforward to monitor their expression in large numbers of samples. If novel genes of unknown function are revealed by such approaches then a host of 'tools' are available for functional analysis; these include deletion and mutation analysis, transgenic models (knockouts), application of the cDNAs to two hybrid analysis to examine protein-protein interactions. and use of expression vectors for production of the encoded proteins for structural and functional analyses.

By comparison, when 2-DE gels are used for protein expression profiling and protein spot changes are detected, there is a good chance that the genes encoding the proteins within spots may be identified by use of matrix assisted laser desorption/ionisation (MALDI) and/or electrospray ionisation (ESI) mass spectrometry (MS) [33, 50, 66, 73, 77, 78, 97–100]. At present the manual nature of many of the steps in the 2-DE process means that it is not generally applicable to confirming the protein expression

a. Now

Gene Identification and Expression Profiling
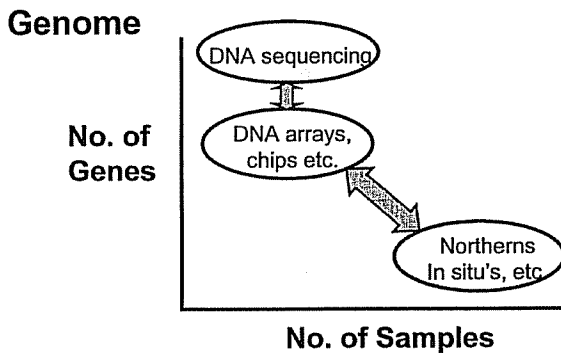
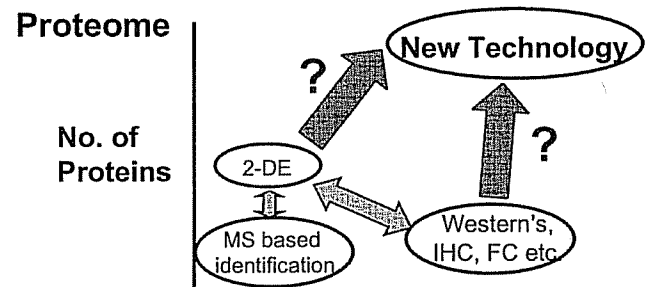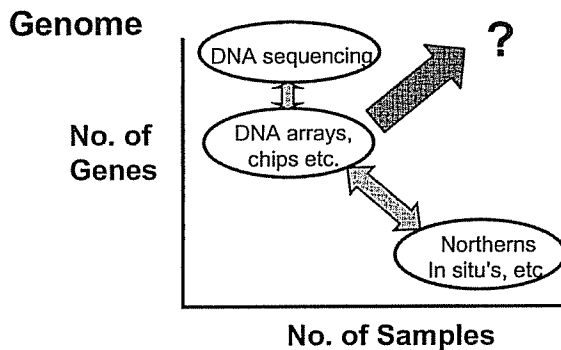### Protein Identification and Expression Profiling
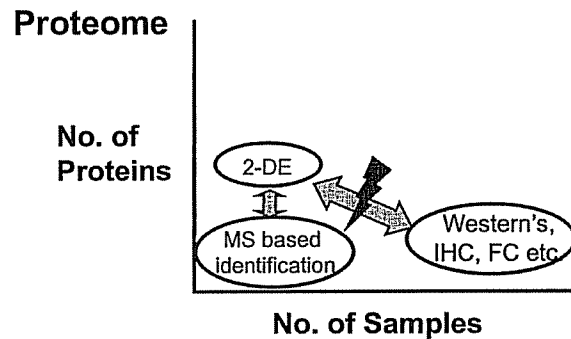
b. Future?

Figure 2. Schematic representation of (a) the current and (b) possible future state of expression profiling. Nucleic acid based methods are currently able to determine the expression (mRNA) of many genes in a small number of samples, but the rapid development of new technologies and automation is likely to enable high-throughput screening of multiple samples. Proteome analyses lag somewhat behind as the current combination of 2-DE and MS allows only a limited number of proteins in an even more limited number of samples to be examined. It is clear that new technologies, possibly in the form of 'protein profiling arrays', are essential if proteomics is to make a significant contribution to functional and structural genomics.

changes on large numbers of samples (Fig. 2a) [101, 102]. Such confirmation of the changes in protein expression revealed by 2-DE is currently most likely to be achieved by Western blotting, immunohistochemistry or other conventional methods to measure protein expression on larger numbers of samples. However, this is difficult because of the relatively limited availability of 'tools' – in this case, antibodies – to the target proteins of interest (Fig. 1 and 2a). Thus, whilst it is possible that antibodies may be available to some of the proteins, it is highly likely that this will be the case for a relatively small minority of the proteins identified.

If available, antibodies to the proteins could also be used for functional analysis, for example, to examine protein-protein interactions, post-translational modifications and to purify the proteins for detailed characterisation. Unfortunately, the amount of protein purified from 2-DE gels is frequently not sufficient to support conventional monoclonal antibody production and screening protocols [103–105]. Anti-peptide antibodies can be readily produced but these may have limited application to native antigen present in tissue sections *etc.* and in some instances it may be important to validate the protein expression changes and their localisation by use of tissue sections [106]. Moreover, whilst significant and exciting advances have been made in the production of phage antibodies [107–113], the selection of such antibodies to native proteins still requires significant amounts of protein and so, to date, has only been achieved using the most abundant protein(s) from 2-DE gels [114]. At this stage in the proteomics process, a move to a genomics and transcriptomics based approach must generally be made. This is in itself not an undesirable strategy: indeed there is likely to be much value in the integration of genomic and proteomic approaches. There will however be some cases such as the analysis of body fluids (blood plasma, urine, cerebrospinal fluid (CSF)) where it is not applicable.

It is apparent that new approaches are required for the production and screening of antibodies that make the process compatible with the amounts of protein purified by 2-DE. We have therefore devised an antibody technology that does not require access to pure protein for either the generation or screening of monoclonal or phage displayed antibodies, and that exploits the power of MS in the screening process (manuscript in preparation). In addition, there is a compelling argument for the development of new technologies capable of supporting rapid, high-throughput analysis of protein expression both for comprehensive analyses and for subsets of 'target' proteins. The importance of proteomics to biological research will undoubtedly drive the development of such new technologies. Below we introduce some current

alternatives to 2-DE based protein expression profiling and then introduce a range of different approaches that may ultimately be assembled to generate separation-independent methods for protein expression profiling.

2 Alternatives to 2-DE for protein expression profiling

The excellent resolving power of 2-DE is achieved through the separation of proteins on the basis of two different physicochemical properties – isoelectric point (p*I*) and molecular weight. This orthogonal approach may also be applied to methods traditionally used as standalone procedures, such as liquid chromatography (LC) and capillary electrophoresis (CE) and methods that potentially support high-throughput resolution and characterisation of proteins in complex mixtures are being developed [115–120]. Thus, size-exclusion chromatography may be used in sequence with reversed phase chromatography [121]; affinity chromatography may be combined with CE [122]; and CE may be linked to HPLC to form the hybrid capillary electrochromatography (CEC) [123].

The use of tandem MS (MS/MS) as a 'detector' following orthogonal separations also adds a further potential separation based on the mass of the proteins and peptides. Moreover, there is currently much interest in the possibility of undertaking direct analysis of relatively complex mixtures of proteins (proteolytically digested) by LC-MS [54, 124–126]. Thus, the sensitivity and mass accuracy of ESI-MS have improved to such an extent that it is now possible to characterise proteins without first purifying them to homogeneity [127, 128]. For instance, the components of the ribosome of *Saccharomyces cerevisiae* and of mammalian interchromatin granule structures have been determined by tandem LC-MS/MS coupled with automated database searching [125, 129]. The searching algorithms are data-dependent so that spectra for individual proteins can be identified amongst the large number of ion peaks and can be subtracted from the next database screen [125, 129]. Such projects are greatly enhanced if there is access to the complete genome sequence of the organism under investigation.

Other recent developments include the use of whole cell stable isotope labelling to support MS-based quantification of proteins and their modification [130, 131] and the use of isotope-coded affinity tags (ICAT) [131, 132]. In the former, cells are maintained in 'light' medium containing a normal range of isotopes, or in 'heavy' medium that has highly elevated levels of a particular isotope, such as ^{15}N. The samples are then pooled, subjected to 2-DE and the spots of interest are digested and analysed by MS: each peptide appears as a doublet with one mass unit diffe-

rence and the relative abundance in the two samples may be determined [130, 131]. Alternatively, isotopic labelling of protein spots may be performed after 2-DE, thereby reducing the requirement for expensive reagents [131]. Once again, the protein spot from one sample is specifically labelled at the *N*-terminus with a 'light' reagent, whilst the protein spot from a second sample is similarly labelled with a deuterated 'heavy' reagent. The digests are combined and the relative abundance of the two proteins determined by the ^{1}H:^{2}H ratio [131].

ICAT is a slightly more sophisticated method of isotopically labelling and isolating peptides. The ICAT reagent is designed to react with sulfhydryl groups and possesses a deuterated linker and a biotin tag. Cysteinyl residues are derivatised with 'light' or 'heavy' ICAT reagents, the samples are combined and digested and the biotin-tagged peptides are isolated by affinity chromatography. The isolated peptides are then identified and quantified by LC-MS/MS [132]. This method has advantages over those requiring 2-DE in that it is suitable for high or low abundance proteins, is independent of the need to label the proteins in intact cells in culture and all steps may be automated for high-throughput analyses.

In order for LC and CE separations to be adapted for high-throughput proteomics, attempts have been made to miniaturise the process on 'chips' [117, 119, 133–136]. These are designed such that multiple analytes may be isolated on a single chip in a parallel fashion, rather than the sequential separations of the orthogonal approaches described above. The development of microfabricated devices for the semiconductor industry has greatly facilitated the design and first stage manufacture of such chips, including the availability of appropriate materials (glass, synthetic polymers) and sample handling devices [117, 133, 137].

Affinity MS is a relatively recent innovation [138, 139] and has become the basis for a form of protein chip. A surface is modified to capture target proteins based on biochemical or intermolecular interactions and bound proteins are then identified directly by MS. Two modifications of this approach are being developed into commercial products for proteome analysis. In surface-enhanced laser desorption/ionisation-MS (SELDI-MS), the target is purified on the SELDI surface *via* chromatographic media such as ion-exchange resin. The SELDI chip may then be inserted directly into a MALDI-MS for analysis of the bound proteins: this technology has been exploited mainly by Ciphergen Biosystems (Palo Alto, CA, USA) (www.ciphergen.com) [140]. The second version of the technology also employs intermolecular interaction between an immobilised receptor and its soluble ligand but the chip

itself is able to detect how much target has been captured [141, 142]. This surface plasmon resonance biomolecular interaction analysis (SPR-BIA) chip may then be analysed directly by MALDI leading to both quantitative and qualitative characterisation of the captured analyte [141, 142].

Whilst the techniques described above, and many others in the pipeline, will no doubt contribute greatly to protein expression analysis and the discovery of novel proteins, some are seeking to develop separation-independent methods for protein expression profiling through application of 'protein arrays' analogous to DNA arrays. Protein arrays to screen antibodies or to directly link proteins back to their appropriate genes, though useful in proteome studies [143–145], are not analogous to DNA arrays as they do not permit a global analysis of protein expression under a given set of conditions. The rest of this review will attempt to identify the potential requirements of the components of 'protein profiling arrays', describe the progress made to date and discuss possible future developments.

3 Protein profiling arrays

3.1 Overview

The key elements in the construction of a protein array will be (i) the generation and isolation of a large repertoire of recognition molecules with which the target proteins will interact, (ii) the means by which the recognition molecules may be immobilised in an array format, and (iii) the detection of the binding of target proteins to the array (see Fig. 3). Obviously, these three requirements need to be both mutually compatible and readily achievable if protein arrays are to become a reality. In the case of DNA arrays, the recognition molecule and the molecules that are recognised are both nucleic acids and hence their physiochemical properties are similar. Also, effective methods for immobilising DNA (to glass or nylon) without affecting specificity have been developed. In terms of detection, radioisotopic or fluorescent tags can readily be incorporated into the cDNA molecules to be detected without unduly influencing the ability of the arrayed DNAs to bind their target sequences.

Clearly, the same properties may not be as easy to achieve for 'protein arrays'. The arrayed molecules could be proteins/antibodies but need not be. Indeed, there are some obvious advantages, such as the stability of the array, to using nonprotein moieties. Immobilisation of the arrayed recognition molecules in a site-directed manner and in such a way that they retain their specificity may prove more difficult to achieve. The incorporation of
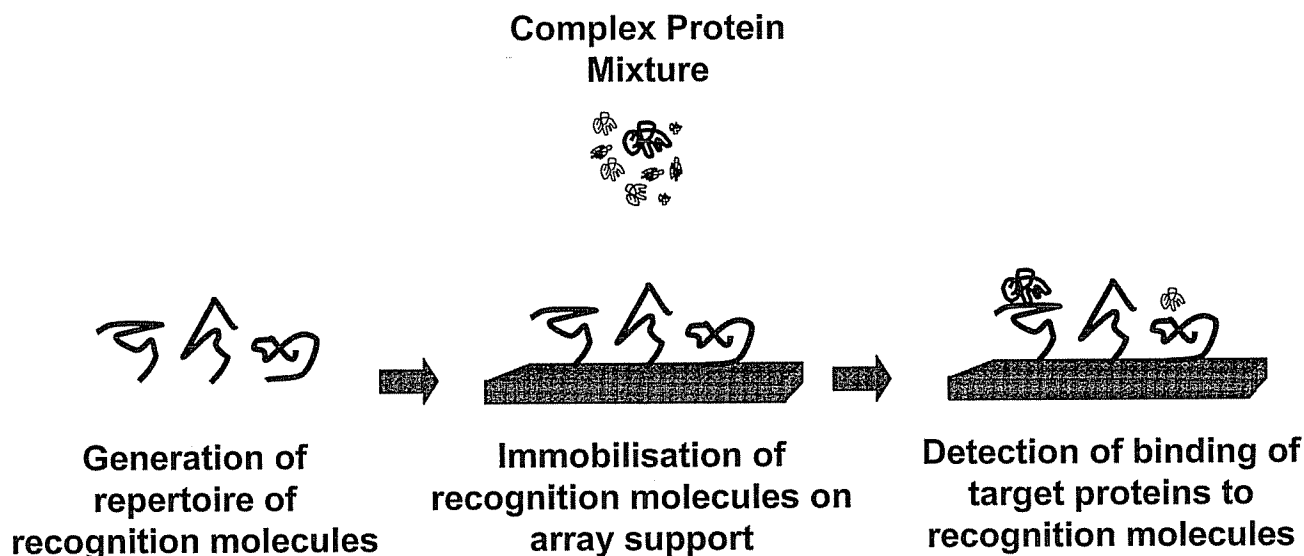
Complex Protein Mixture

| Generation of repertoire of recognition molecules | Immobilisation of recognition molecules on array support | Detection of binding of target proteins to recognition molecules |

Figure 3. Schematic representation of the components of a protein array. The recognition molecules should be capable of binding individual protein moieties or a range of known protein moieties with appropriate affinity and specificity, *i.e.* they should be capable of recognizing their protein target(s) selectively when a protein mixture is presented. The recognition molecules must be immobilized to a solid support such that their ability to bind to the target protein(s) is not compromised. Once target proteins are bound to their cognate recognition molecules quantitative detection of the binding must be achieved with appropriate sensitivity and dynamic range.

radioisotopic or fluorescent tags into the protein molecules to be detected must be stoichiometrically reliable and must not influence the ability of the proteins to be bound specifically by the arrayed recognition molecules. It could be argued therefore that attempts to make protein arrays by mimicking DNA arrays may not prove to be effective and that a more radical approach to the design and development of protein arrays may be required. To stimulate such developments we describe a range of possibilities for the generation and immobilisation of protein recognition molecules (PRMs) and the detection of bound target proteins. We do this without attempting to make any judgement on their suitability or otherwise for incorporation into 'protein profiling arrays', in part because the most suitable format for such arrays is likely to remain unclear until further enabling science has been undertaken.

3.2 Protein recognition molecules

Ideally, the PRMs used to support the development of protein profiling arrays would be chemically robust for stability, and capable of binding their target protein molecules with high efficiency and with suitable discriminatory specificity. Thus, they must be able to recognise an individual protein moiety (or known combination of protein moieties) from a complex mixture of proteins. Clearly, it

would also be desirable to have a well-characterised and comprehensive repertoire of PRMs such that all potential protein moieties, including all protein precursors and post-translationally modified forms of proteins, could be discriminated. Even the most optimistic of readers will appreciate that this is a tall order indeed. However, it should also be apparent that more modest repertoires of characterised PRMs, provided they are directed against proteins of functional importance, afford the opportunity to develop valuable protein arrays for diagnostic and prognostic screening purposes.

The most obvious choice of PRM is an antibody, as its primary biological function is molecular recognition. There are also well-established methods for the production of both polyclonal and monoclonal antibodies [104, 105]. However, it is important to appreciate that the demands placed on the generation of antibodies to support the production of protein arrays may be significantly more stringent than for the conventional applications of antibodies. Thus, one must be able to generate and select the antibodies in cases where the target protein is impure or rare, for instance, low-abundance proteins taken from 2-DE gels. At present most antibody screening methods (i) require relatively large quantities of protein, and (ii) do not automatically result in the selection of antibodies that specifically recognise the protein(s) of interest when pre-

sent in a mixture. Antibodies specific for all the post-translationally modified forms of the protein must also be represented. Furthermore, it would be convenient if all the antibodies on a single array bound to their targets with similar kinetics (affinity, association constant, dissociation constant) under a given set of conditions. If arraying is successfully achieved for all the represented antibodies, then the array must be suitable for storage in a manner that is convenient to access whilst maintaining the reactivity of the antibodies. This might involve lyophilisation or storage in liquid nitrogen. Clearly, it is difficult to envisage the generation of a comprehensive antibody array, that is one which would support the expression profiling of several thousand proteins, with the technologies available today. However, it is perhaps not so difficult to envisage how a more modest array could be developed.

Phage displayed antibodies provide an alternative source of PRMs. Phage display technology is based on bacteriophages that have been genetically modified to act as efficient transporters of DNA inserts into their bacterial hosts, where the phages are amplified and packaged to express foreign proteins of interest on their surfaces, including short chain variable fragments of immunoglobulins (scFv) [110, 112, 113, 146–149]. Such phage-displayed antibodies may be affinity selected and the clones amplified to produce monospecific antibodies: these may then be 'matured' to enhance properties such as affinity and specificity for the target [110, 113, 148, 149]. In addition to some of the libraries exhibiting problems with selection of high-affinity scFv's [111], many of the same difficulties that apply to arraying conventional antibodies may also apply to phage displayed antibodies.

One solution that may overcome some of these difficulties is the exploitation of a PRM that is not itself a protein. Aptamers are single-stranded oligonucleotides that express high affinity for conformation-dependent molecules such as proteins [150, 151]. They have several advantages over their antibody counterparts: libraries of nucleic acids may be manufactured to order; they are readily immobilised to a solid support; and they are considerably less labile than proteins. Despite these obvious benefits, there have been remarkably few publications exploiting aptamer libraries since they came to public attention approximately 10 years ago [150]. This may be due to the multiple rounds of screening and amplification, generally between 5 and 10, that are required to select each aptamer, or to the fact that the technolgy seems to be more suited to isolation of small planar molecules with little hydrophobicity [152]. In addition, the aptamers emerging during a screen tend to recognize the more abundant or more recognizable target molecules, rather than perhaps the more interesting ones. It has also become appa-

rent that the sites on the aptamers responsible for interaction with the ligand are the same as those responsible for determining the shape of the aptamer, reducing the chances of producing an antibody-like molecule with a generic backbone and a binding region that can be varied by mutation [152]. Finally, nucleic acid aptamers, particularly RNA aptamers, are susceptible to nucleases that are likely to be present in complex protein mixtures. Despite these drawbacks, the technology may have potential for future development and incorporation into protein profiling arrays.

The potential difficulties with exploiting 'biological' molecules as PRMs, has led some to explore the use of molecularly imprinted polymers (MIPs) as an alternative means to perform affinity capture [153–160]. The three-dimensional shape of a target molecule may be mimicked in such synthetic supports to generate a binding surface that is resistant to biological attack and is largely unaffected by acidity, temperature and other experimental conditions [157–159]. Most of the MIPs generated to date have been used for detecting small compounds present in environmental samples or biological fluids, but the potential exists to develop them as protein binding surfaces. There remain many hurdles to overcome, such as the degree of specificity that could be obtained by a MIP given that it could potentially interact with all the epitopes of a particular protein moiety (in antibody terms, it is not monoclonal). As with conventional antibody production, it will also be necessary to have access to purified protein in order to generate the MIP. There may, however, come a time when it may be possible to produce MIPs based on computer-generated 3-D structures of proteins via laser etching or self-assembling polymer.

3.3 Arraying/immobilisation of PRMs

The means by which the PRMs are arrayed and attached to the array surface will obviously depend upon their nature. The basic requirements of the arraying procedure are spatial definition, reproducibility, stability and retention of high specificity and affinity.

3.3.1 Arraying of nucleic acid PRMs

Methodologies already exist to array nucleic acids but these are constantly being upgraded in an attempt to increase the number of targets per unit area, thereby increasing the data generated per array or chip. The simplest, and therefore probably the most accessible arrays, comprise cDNA inserts or PCR products that have been 'dot-blotted' onto a nylon membrane of approximately the size of a 96-well plate. The DNA binds strongly to the membrane and in a manner that enables its hybridisation

to single-stranded probes [161]. Preparation of these arrays has been both automated and miniaturised so that up to 10 000 cDNAs may be spotted onto a membrane the size of a postage stamp [95]. The arraying protocol has been modified through the use of glass microscope slides as supports for the DNA instead of membranes. The glass is silanated and the cDNA is spotted onto the surface in a grid pattern using a computer-controlled tridirectional robot to which capillary-tipped pens are attached [93]. The DNA is then immobilised by exposure to UV light. Glass microarrays such as these are able to represent between 5 000 and 10 000 genes per cm^2 [82, 95].

The most sophisticated method developed to date for arraying nucleic acids is the *in situ* synthesis of oligonucleotides such that up to 64 000 'features', each containing several million oligonucleotides, may be represented on a small glass chip. The oligonucleotides are synthesized from modified photo-labile deoxynucleosides that polymerise on exposure to uv light. The sequence of the DNA is directed by masking from irradiation areas of the chip where the available nucleoside is not required [82]. A recent modification of this technology involves virtual masking in which a computer describes the areas of the chip to be irradiated [96].

Each of these technologies could be adapted as a platform for an array for protein expression profiling in which the PRMs were nucleic acid aptamers. The selected nucleic acid sequences could be spotted onto a membrane or synthesized *in situ*. Membranes are accessible and manageable, but suffer from potential background problems and relatively large surface areas. Glass chips are less susceptible to high backgrounds, would require considerably less target protein mixture, and are more amenable to automation. Thus, the future prospects for immobilising aptamers for protein arrays mirrors the current position for gene expression arrays.

3.3.2 Arraying of proteinaceous PRMs

Proteinaceous PRMs, be they monoclonal antibodies, phage displayed antibodies or other polypeptides, will probably need to be attached to a suitable surface in a spatially defined manner. It is likely that noncovalent interactions would be too weak to prevent some loss of PRM during protein expression profiling, but the PRMs are less likely to be affected in terms of functionality by noncovalent immobilisation. The use of covalent interactions in the manufacture of the array would make it more robust, but unless carefully controlled might affect the affinity, specificity or efficiency of the PRM-protein interactions.

In the case of monoclonal antibodies, some means to attach the molecules *via* their Fc portions, without affecting the conformation of other regions of the molecule, is likely to be required. If allowed to attach randomly to the support, a percentage of the antibodies to be immobilised may fortuitously align themselves correctly, and in some cases this has been reported to have little effect on overall binding capacity [162]. Sensitivity of target protein detection is likely to be an issue with such antibody arraying conditions indicating that orientated binding may be required. Orientation of immunoglobulins is often achieved through the use of Protein A/G that has the required specificity for Fc regions, but such an approach to generating an antibody array does not seem very elegant. However, several modifications of this standard immobilisation procedure are being investigated. For instance, Protein A has been engineered to express five copies of the immunoglobulin G binding domain plus a cysteine residue at the *C*-terminus, the latter to allow strong binding to gold immobilisation surfaces [163]. Others have exploited the carbohydrate moieties on the Fc portion of the antibody to orientate them using carbohydrate affinity gel matrices [162].

Many immobilisation strategies rely on noncovalent interactions between the protein to be immobilised and the array surface. These may be based on the affinity of the base layer for the PRM, as in the case of Protein A, or on hydrophobic, ionic, or van der Waal's interactions between the PRM and the absorptive surface [164, 165]. In order to covalently bind the PRMs, metal or silica surfaces are photolithographically etched and proteins are attached *via* a chemical linker such as aminosilane capable of forming covalent bonds with both array surface and PRM [165]. Alternatively, substances such as glutaraldehyde and *N*-succinimidyl-4-maleimidobutyrate may be used as cross-linkers [165–167].

Notwithstanding these considerations, attempts to array proteinaceous PRMs have been initiated successfully [165]. In one such, acrylamide gel has been photopolymerised into a grid pattern using photolithography, followed by transfer of the PRM (IgG or BSA) to the gel surface by a multipin device in much the same way as the cDNA microarrays were prepared [135]. In later systems, the acrylamide was replaced by an absorptive protein layer such as streptavidin. The streptavidin was micropatterned using ink-jet printing techniques then exposed to biotin followed by the protein to be adsorbed [168]. Alternatively, a streptavidin layer was overlaid with photobiotin and the grid generated by exposure to UV light [168]. Both of these techniques required multiple layers to be deposited sequentially and were prone to poor specificity because of cross-contamination of the squares of the

grid [168]. However, the fact that commercially available ink-jet printers may be modified to generate arrays on cellulose paper makes them accessible to any interested party [169].

One of the most recent microfabrication methods to have been proposed for patterning biomolecules is termed 'soft-lithography' [168, 170–172]. This comprises an elastomeric material of 'hydrogel' that can be moulded to form stamps or channels for the transfer of proteins to appropriate surfaces. For instance, Gaber and colleagues [168] have used drawn-out capillary tubes filled with freeze-dried disaccharide acrylate polymers that, when exposed to aqueous protein solution, reswell and form a protein-saturated nib. The method is said to be suitable for arraying small, well-defined areas of delicate biomolecules with relative ease [170–172]. Electrospray deposition may also be effective for arraying delicate biomolecules since it only requires a support that is slightly conductive, such as damp membrane, to achieve immobilisation [173]. Whether the arraying methods described above will be as versatile and practicable as those currently used to generate DNA arrays remains to be seen.

3.3.3 Arraying of synthetic PRMs

The development of MIPs is still at an early stage compared to organic PRMs, yet they would have many advantages over proteins and nucleic acids in an array format. There would be no difficulty with array longevity nor would the ability to capture target be compromised by assay conditions. Combinatorial libraries of MIPs are already under construction, and the process has been semi-automated by the use of liquid handling robots [155], and miniaturised to produce microcolumns of 1mm internal diameter [156].

4 Detection

The final stage in screening a protein profiling array is the detection of bound targets in a manner that is compatible with the requirement for sensitivity and speed. The emergence of biosensor technology has encouraged multidisciplinary collaborations between biologists, chemists and physicists [174], and it is likely that this technology will become applicable to protein arrays for highly parallel protein expression profiling. Biosensors comprise an affinity molecule coupled to a physicochemically sensitive layer that is affected when target molecule binds to the surface [175, 176]. Changes in the sensing layer, such as emission of light, heat, electrons or ions or a change in mass, are transduced into an electrical signal that provides a measure of the amount of target bound [175–178].

Perhaps the most straightforward approach to the detection of bound target would be to capture the target proteins with the immobilised PRMs, then detect bound targets with a second, fluorescently labelled detection molecule as in conventional 'sandwich' techniques. This approach has been used in limited array formats on waveguide biosensors to capture multiple analytes simultaneously and detect them with a CCD camera [179, 180]. Whilst potentially suitable for some diagnostic tests, this format is not likely to be applicable to protein profiling arrays due to the amount of processing required for what is, in effect, a mini-ELISA, and the requirement for optically transparent samples [177]. This means that whole blood, for instance, could not be analysed. An alternative that has been used to detect explosives utilised displacement of fluorescently-labelled target from an antibody-coated probe when the probe was exposed to a test sample [181]. Again, transferring this method to an array format for rapid, parallel analyses would be problematic.

It may be possible to label all the proteins in a target mixture with a fluorescent tag, such as fluorescein isothiocyanate, dichlorotriazinylamino-fluorescein or dansyl chloride [76], and to visualise the array using microscopy, phosphorimaging or biosensor readout. Dual labelling of target DNA is used in microarray hybridisation to determine differential mRNA expression [182]. A similar dual-labelling strategy has been described for proteins in which samples are tagged with either propyl-Cy3 or methyl-Cy5 prior to simultaneous visualisation of the proteins on the same 2-DE gel [183]. Extending this to arrays may be technically difficult as labelling with fluorescent tags involves chemical modification of the proteins and may adversely influence the interaction of the proteins with their respective PRMs. In addition, the incorporation of label into proteins is much more difficult to control than incorporation of label into nucleic acids so that accurate quantification of labelled protein may be difficult to achieve. Finally, the sensitivity of fluorescence detection is such that a large-format array may be required and clearly this is not in keeping with the current trend of miniaturisation.

Other recent developments in detection methods have concentrated on a means to directly detect individual PRM-target molecular interactions. One such method is atomic force microscopy in which a cantilevered probe directly impacts on the protein surface and measures the relative depth of the protein layer [184–186]. The drawbacks of this method for array analysis include the likely difficulty with automation, the sequential rather than parallel nature of the readout and the possible damage to the array [165]. However, the basic cantilever-driven detection method has been adopted by naval scientists in a force amplified biological sensor (FABS) [187, 188]. In

this case, the capture molecule is covalently bound to the cantilever, the target is allowed to adsorb, a second antibody labelled with a magnetic sphere is applied and the movements generated by a magnetic field are measured [189]. The group is attempting to adapt the technology to a high-throughput format by photopatterning micrometer scale magnetic field sensors and developing an array detector to count the number of beads remaining after a magnetic force has been applied [187, 188].

As described in Section 2, an approach that utilises a mass spectrometer as the detection device is SELDI-MS. The basic premise of affinity capture followed by MS has been refined by Ciphergen in particular to form the basis of a protein chip [140]. The target may be captured either by a range of diagnostically significant antibodies, or by a series of chromatographic matrices, and analysed directly by MS. However, unless the chip is subjected to enzymatic or other modification, the information gained in this manner is restricted to molecular weight of the complete target, and it is not quantitative. Whilst being a potentially helpful diagnostic tool, it may have limitations as a proteomics tool in cases where the relative abundance or post-translational modifications of proteins are significant. The combination of an SPR biosensor with on-chip MALDI analysis will overcome some of these problems [141].

Another direct measurement of antibody-antigen interaction is through the use of piezoelectric devices in quartz crystal microbalances (QCMs). These biosensors are able to transduce molecular interaction events into electric signals *via* a quartz crystal coated on both sides with gold, silver, aluminium or nickel [190, 191]. The crystal resonates at a set frequency that is dependent on the mass of the crystal and on the mass of proteins layered on the metal surface. The change in mass that occurs as an immobilised antibody recognises an antigen in solution causes a change in resonance frequency that can be recorded as an electrical signal and extrapolated to determine the amount of antigen bound [191–192]. This simple and direct measurement of biomolecular interaction has been incorporated into various biosensors for diagnosis, environmental screening and drug screening [166, 192]. It has limitations as both a biosensor and as the basis of a protein profiling array, the main one being its inability to measure small changes in mass. Thus it is only suitable for target proteins with relatively high molecular masses, the level of sensitivity at present becoming unacceptable below approximately 10kDa [191]. Modifications of the direct readout involve labelling the target or employing a labelled secondary antibody in order to increase the change in mass on antibody-antigen binding [191], but

this as already discussed introduces unacceptable complexity in terms of probing a protein array.

A detection method that has many similarities to QCM is SPR, but instead of measuring changes in oscillations, it measures changes in refractive index on target binding [190]. A glass slide is coated with metal, and then a self-assembled monolayer displaying active esters is cross-linked to the protein to be immobilised. Binding of target to immobilised protein increases the mass at the chip surface, that in turn alters the refractive index of the glass and this change may be related to the quantity of protein bound. SPR has also been used in experiments to map epitopes, and to examine the affinity and kinetics of antibody-antigen interactions [141, 190]. The technology has shown sufficient promise for it to be taken up commercially by Biacore, (Biacore, Uppsala, Sweden (www.biacore.com)) and Affinity Sensors, (Cambridge, U.K. (www.affinity-sensors.com)) [141]. The BIAcore system simultaneously analyses four samples as they flow over the sensor chip and is able to accommodate up to 192 samples in one run. In addition, the BIAcore chip may be used directly as a MALDI target for MS analysis [178] and as such has been evaluated as a method for epitope mapping and as a bioanalytical tool [141].

One of the latest developments in biosensor technology is the use of ion channels to detect antibody-antigen binding [193, 194]. This biosensor comprises a gold electrode attached to a lipid bilayer that contains immobile gramicidin ion channels within the layer adjacent to the electrode, and mobile gramicidin channels within the outer lipid layer. The mobile ion channels are biotinylated, as are antibody fragments, and they are linked together by streptavidin intermediates. Some of the immobile membrane spanning lipids are also biotinylated and therefore bind antibodies. In the absence of antigen, the mobile ion channels are free to move about and occasionally associate with the immobile ion channels resulting in a flux of millions of ions detectable by the gold electrode [194]. In the presence of antigen, the mobile ion channels are anchored to the immobile membrane spanning lipids *via* an antigen bridge and prevented from associating with the channels in the inner lipid layer. There is therefore a reduction in the flow of ions proportional to the amount of antigen in the sample [194]. The sensitivity of the biosensor is reported to reach subpicomolar levels, and the ability to detect ion fluxes through individual channels indicates that it is possible to miniaturise the electrodes. Despite the similarity to ELISA methods, the authors state that there is no requirement for washing and incubation steps as the capture and detector antibodies are pre-assembled, rather than being added sequentially [194]. The biosensor may suffer from leakage of molecules

through the membrane, insufficient level of ions in the reservoir beneath the bilayer, and poor longevity [177]. The disadvantages in terms of an array technology are the relative complexity of the detection method and the requirement for chemically modified antibodies.

There are other forms of biosensor in development that utilise alternative physicochemical properties to transduce the binding signal [177, 178]. For instance, enzyme-inhibitor and enzyme-substrate interactions have been detected using measurements of current (amperometric) and of voltage/pH (potentiometric) [195, 196], whilst a capacitive biosensor was used to detect hybridisation of DNA [197]. Although electrochemical sensors are widespread in diagnosis of diseases involving enzymes, such as diabetes [177], there are fewer reports in the literature of these methods being used for antibody-antigen interaction studies.

5 Does miniaturisation matter?

When discussing a format for protein arrays, or indeed nucleic acid arrays, it is generally assumed that miniaturisation will be both necessary and optimal. There are certainly advantages in reducing the size of the array in terms of the quantity of immobilised capture molecule and of sample required for analysis. A high concentration of sample protein in a small volume should increase the speed at which target binds to its cognate partner thereby decreasing incubation times. The use of microarrays will have the knock-on effect of reducing the storage and packaging facilities required and the size of the instrumentation required for analysis of the arrays. The potential ability to measure individual intermolecular interactions means that the need for sensitivity does not impact on the miniaturisation issue.

There are, however, potential disadvantages to miniaturisation. The volumes of reagents used in microarray formats will be small and subject to handling errors unless the resolution and reproducibility are strictly regulated. Lower volumes and shorter incubations will also be more susceptible to variations in reaction conditions and will therefore need to be tightly controlled. Finally, the dynamic range of proteins in biological samples, which is estimated to be at least seven orders of magnitude [43], will potentially be a problem for any protein profiling array and may be further compromised by miniaturisation.

6 Conclusions

There is little argument that a robust, high-throughput method of profiling the expression of proteins to comple-

ment or replace 2-DE would make a dramatic impact on proteomics. Many of the technologies that could be applied to the generation of protein arrays are either already established or are in active development. Thus, there are multiple means by which PRMs may be generated and immobilised and bound targets detected, some of which we may have overlooked. However, the integration of these into a robust and reliable platform is likely to require a multidisciplinary approach that brings together chemists, material scientists and biologists and, of course, time. Some commercial organisations have already embarked on this route and we await the results of their efforts with great interest.

7 References

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